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SLIME GROWTH EVALUATION OF TREATED PULP MILL WASTE

A. W. Anderson, et al

Oregon State University

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Slime Growth Evaluation of Treated Pulp Mill Waste

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10 Author(s)

A. W. Anderson

G. A. Beierwaltes

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27 Abstract

The main objectives were to evaluate the slime growth potential of pulp mill wastes treated by various methods of biodegradation. Wastes were tested both before and after secondary treatment in order to determine the type of biodegradable material present in the influent, determine the extent of fermentation during treatment, and the amount of biodegraded fermentables discharged in the effluent. The studies were carried out in an effort to define total carbon, readily fermentable carbon, and to design a reasonably accurate and sensitive method for predicting adequate water quality presently measured by BOD.

Gas chromatographic analysis of monosaccharides, total carbon, and continuous culture proved to be effective analytical procedures. These techniques showed that Sphaerotilus natans would grow in treated effluent, that residual sugars in the treated effluent could be detected easily by GC, that total solids and sugar concentrations parallel influent flow rate, that during influent surges, effluent sugar concentrations are high enough to support slime growth, that weekly variations were evident, and that differences between the two treatment ponds could be observed. These variations could not be easily observed by the usual BOD determinations. Thus, adequate biotreatment could be determined by these three methods. (There are 11 references).

Abstractor	Institution
A. W. Anderson	Oregon State University

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SLIME GROWTH EVALUATION OF TREATED PULP MILL WASTE

by

Department of Microbiology
Oregon State University
Corvallis, Oregon 97331

for the

ENVIRONMENTAL PROTECTION AGENCY

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ABSTRACT

The main objectives were to evaluate the slime growth potential of pulp mill wastes treated by various methods of biodegradation. Wastes were tested both before and after secondary treatment in order to determine the type of biodegradable material present in the influent, determine the extent of fermentation during treatment, and the amount of biodegraded fermentable compounds discharged in the effluent. These studies were carried out in an effort to define total carbon, readily fermentable carbon, and to design a reasonably accurate and sensitive method for predicting adequate water quality presently measured by BOD.

The gas chromatographic analysis of monosacchardies along with total carbon and continuous culture proved to be a very effective analytical procedure. By the use of these techniques, it was possible to prove beyond a doubt that Sphaerotilus natans would grow in the treated effluent, that the residual sugars in the treated effluent could be detected easily by gas chromatography, that weekly variations in treatment were noted, and that differences between the two treatment ponds could be easily observed. These variations could not be easily observed by the usual BOD determinations.

These techniques make it possible to determine when bio-treatment is adequate, thereby aiding in the design of proper aeration equipment, better treatment pond design, and a means of monitoring rapidly changes in influent composition.

Our findings indicate that pond II at the Lebanon treatment plant is more efficient biologically than pond I, that when the influent flow rate is high, the total solids and sugar concentrations are high, and it is during these surges that sugars appear in the treated effluent at levels which will easily support slime producing micro-organisms in the outfall and receiving streams.

This report was submitted in fulfillment of Project No. 12040 DLQ under the sponsorship of the Environmental Protection Agency and Oregon State University.

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SECTION I

CONCLUSIONS

1. Plate counts of Sphaerotilus as an indicator organism, could be useful in establishing water quality criteria where organic pollution is suspected.

2. Total growth yields of Sphaerotilus obtained from continuous cultures might provide a suitable means for assessing the efficiency of different treatment methods.

3. BOD determinations alone do not give an accurate picture of the secondary treatment efficiency at the Lebanon mill.

4. Continuous culture studies combined with total carbon, and total monosaccharide determinations were found useful in assessing adequate bio-treatment.

5. Increased aeration of the Lebanon mill influent results in total bio-oxidation of the readily fermentable compounds, thus reducing or eliminating slime growth potential in the effluent.

6. Total carbon and total sugar analyses indicate that pond II is more efficient than pond I for secondary treatment.

SECTION II

RECOMMENDATIONS

Several possibilities exist which could improve secondary treatment, thus reducing slime growth potential in the receiving stream at the Crown Zellerbach mill in Lebanon, Oregon:

1. Increase aeration by increasing aerating capacity.

2. Change placement of aerators so that influent short circuiting would be avoided.

3. Operate ponds in series rather than parallel, thus insuring minimal chance for short circuiting. (The ponds were not operated in series during the period of sugar determinations.)

4. Redesign the treatment facilities in order that retention times could be adjusted to correspond with the BOD surges in the influent, or conversely, so that the inflow rate is such that BOD levels remain relatively constant.

5. Remove biomass from the effluent and thus remove future possible nutrients for slime growth.

6. Utilize total carbon and monosaccharide analyses either in place of, or in conjunction with BOD determinations of treatment efficiency.

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SECTION III

INTRODUCTION

Project Plan and Specific Aim: The introduction of sulfite waste liquors into streams often leads to the development of unsightly masses of biological slime dominated by Sphaerotilus. This organism has the ability to extract sufficient nutrients from large volumes of water containing very low levels of wastes. As a result, it has developed in areas that are judged to be in excellent condition with respect to dissolved oxygen and BOD. For example, it has been shown (10) that Sphaerotilus is able to grow in sulfite waste liquors at BOD levels as low as 0.06 ppm. These characteristics of Sphaerotilus add to the difficulty of developing adequate waste treatment procedures for controlling slime and in establishing suitable water quality criteria.

Problems arising because of slime growths in streams have been reviewed by many authors and will not be recapitulated here (4,5,7,9). It is sufficient to note that masses of Sphaerotilus often interfere with the aesthetic quality, uses of water and the ecology of a stream.

A number of studies have been carried out on the nutrition and physiology of Sphaerotilus to determine the specific conditions that promote its development. These studies have shown that it is capable of growing on a wide variety of carbon and nitrogen sources under diverse conditions. The major constituents in spent sulfite liquor which promote the growth of Sphaerotilus include the hexose sugars and acetic acid. The quality of the receiving water also plays a role in the stimulation of Sphaerotilus in streams.

One approach for controlling the development of Sphaerotilus in streams receiving pulp mill wastes has been to employ biological methods for treating wastes before release into a receiving stream. The activated sludge process has been found to be effective in reducing the slime potential of the wastes although growth of Sphaerotilus is not completely prevented by this treatment (2).

A study was made by personnel of Crown Zellerbach Corporation to improve the effectiveness of waste treatment for controlling biological growths in streams receiving pulp mill wastes. Facilities were prepared at the Lebanon, Oregon plant of Crown Zellerbach Corporation for a variety of treatment schedules and environmental parameters to test the efficiency in the treatment of pulp mill wastes. Two aeration basins were available for treating the wastes. The following conditions were investigated by Crown Zellerbach.

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a. The optimum phosphorus concentration for waste treatment.

b. Differences in the effectiveness between series and parallel operation of the aeration basins.

c. The effect of BOD load on BOD reduction.

d. The effect of retention time on BOD reduction.

e. The influence of treatment temperature on efficiency.

f. The effect of recirculating treated wastes on treatment efficiency.

In addition to the above conditions, a variety of chemical analyses were performed by personnel of Crown Zellerbach on samples taken from inlets and outlets of the aeration basins to assess the effectiveness of the various treatment. These analyses include determinations of BOD, COD, dissolved oxygen, pH, total organic carbon, reducing sugars, total nitrogen, ammonia nitrogen, and ortho-phosphate.

In addition to a chemical analysis performed by Crown Zellerbach on the treated wastes, the Microbiology Department Water Laboratory at O.S.U. evaluated the growth promoting properties of the wastes in terms of slime production. The objectives were to provide the necessary microbiological investigations to accompany and be closely coordinated with those being carried out by the Central Research Division of Crown Zellerbach Corporation.

The objectives as planned were designed to determine the effectiveness of the presently used experimental treatments of pulp mill waste. The investigation encompassed the following:

a. Assess the growth supporting properties of paper mill waste water using a mixed culture (obtained from the mill fermentation pond) and a pure culture of Sphaerotilus natans. This includes both the influent and effluent.

b. Develop techniques for determining qualitatively and quantitatively which type of compounds have been removed and which have not.

c. Determine what effect engineering changes in the treatment system have on the biological removal of fermentable wastes.

It was anticipated that this investigation would assist engineers in designing a better and more economical treatment system for removing biodegradable compounds, many of which cause pollution of Oregon streams and the surrounding environment.

SECTION IV

MATERIALS AND METHODS

Initially it was planned to assess the growth promoting potential of the treated pulp mill wastes using two approaches. One of these involved the use of six experimental streams available at the Lebanon site of Crown Zellerbach Corporation. These streams were to be supplied with fresh water which could be used in preparing various dilutions of both treated and untreated wastes for study.

Slime production in the streams was to be assessed by several means. These included a plate count procedure and a slide assay technique. Attempts to correlate these approaches with the biomass approach used by Crown Zellerbach personnel were planned.

Plate Count Procedure: One-fourth strength Cytophaga (1/4 CA) was selected as the medium of choice for recovering Sphaerotilus from water. This medium contained the following ingredients:

Tryptone	0.125%
Yeast extract	0.125%
Beef extract	0.005%
Sodium acetate	0.005%
Agar	1.5%

Distilled water was used to prepare the medium since it was found that, at times, tap water was toxic to Sphaerotilus.

The plating procedure consisted of spreading a known quantity of water on the surface of a 1/4 CA plate. Bent glass rods were used to distribute the water samples on the plate. To prevent pseudomonad and motile eubacterial colonies from spreading over the surface of the agar and interfering with Sphaerotilus growth, the plates were dried at 37°C for 24 to 48 hours before inoculation. By use of a dry agar surface it was possible to plate up to 0.5 ml of water directly. The plates were incubated at 18°C.

Sphaerotilus colonies appeared on the plates after 48 to 72 hours incubation. The organism was readily recognized on the plates by the appearance of the colonies. Under 15x magnification, colonies of Sphaerotilus on 1/4 CA were long and filamentous with a coarse texture and were generally branched. A high degree of refractility was also

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apparent in the colonies. A colony with some similarity to Sphaerotilus was produced on 1/4 CA by a Bacillus. It was possible, however, to differentiate the two organisms by the fact that the Bacillus colony exhibited filaments with a folded appearance (similar to switchbacks). Also, the Bacillus colonies were less refractile than those formed by Sphaerotilus. Questionable colonies could readily be identified by examining wet mounts of colony material under phase contrast for the presence or absence of sheaths.

Slide Technique: Clean glass slides were immersed into the stream for a period of time and the number of Sphaerotilus filaments which attach to the glass counted.

Another approach for monitoring slime production involved the use of the continous culture procedure. This method consisted of the addition of filter sterilized pulp mill wastes at a constant rate to a continous culture vessel inoculated with Sphaerotilus, and a mixed culture obtained directly from the treatment ponds. The continuous fermentations were carried out in 500cc Bellco spin flasks (Bellco Glass Co., Vineland, N.J.). The culture medium (influent, effluent or fortified substrate) was fed into the flasks using a polystaltic pump (Buckler Model 2-6100, 1327 16th Street, Fort Lee, New Jersey, 07024).

All samples were adjusted to pH 7 and filter sterilized, several hours after collection. The turn-over rate of medium in the culture vessels was varied from 24 hrs to 7 days and air was usually supplied at a constant rate of 2.0 CFH. The flasks were usually run simultaneously as follows:

Continuous culture I: mixed culture from Pond II inoculated into sterile influent.

Continuous culture II: a pure Sphaerotilus culture inoculated into sterile influent.

Continuous culture III: a mixed culture from Pond II inoculated into sterile effluent.

Collection and Treatment of Samples

Grab Samples: Weekly 15-20 liter grab samples were collected directly from the influent and effluent flow at the secondary treatment ponds. Time of collection, plant treatment, pH, and ambient pond temperature were recorded. These samples were subjected to the following treatment:

a. Centrifuged on a Sharpless continuous centrifuge (Model TIP) to remove the heavier particulate materials

(within several hours following collection.)

b. Total suspended solids were determined by drying the centrifugate wet solids at 105°C under low vacuum for 24-48 hours, and equating this on the basis of mgm/ml (ppm).

c. When needed for continuous culture studies, the liquid was filter-sterilized by using an industrial Sietz filter, following centrifugation. This filter sterilized liquid, influent or effluent, was used as substrate for the growth of a mixed culture and of Sphaerotilus natans in continuous growth fermentation flasks.

d. 150 ml portions of the centrifuged samples were treated with $BaCl_2$ until no more precitate formed. The precipitate was removed by centrifugation and the supernatant lyophilized. The dried samples were stored for later use in thin layer chromatography.

e. The above treatment from steps 'a' through 'd' was also carried out on the test samples of influent and effluent collected from the spent substrate following continuous culture growth in the laboratory.

24-hr Composite Samples: After setting up refrigeration units in November, 1970, one liter, cooled (2-9°C), 24-hr composite samples based on flow rate of influent and effluent were collected at weekly intervals. These samples were used to quantitate carbohydrates, free volatile acids, alcohols, and total carbon present.

Thin Layer Chromatography

For thin layer chromatographs, carbohydrates were extracted from the lyophilized solids with 95 percent ethyl alcohol. This decreased the interference of lignosulfonates and wood pigments resulting in the development of a better thin layer chromatogram. The alcohol extract was applied to thin layers of Silica Gel G (30gm) impregnated with the following solutions (60ml): 0.02M sodium borate buffer; 0.02 M sodium acetate; 0.1 M sodium bisulfite; 0.02 M boric acid; water. The solvent mixtures used to develop the chromatograms are shown in Table 1. Visualization agents employed were mixtures of conc H_2SO_4/ dichromate (charring), conc H_2SO_4/anisaldehyde (specific color development), conc H_2SO_4/napthoresorcinol (specific color development) and anilin/diphenylamine (specific color development).

Table 1

Solvent mixtures used to develop thin layer chromatograms for the detection of pentoses and hexoses from aeration pond influents and effluents.

No	Solvent Mixture	Composition (ml)
1	Acetone - Water	90:10
2	Chloroform - Methanol	60:40
3	Benzene - Acetic Acid - Methanol	20:20:60
4	n-Butanol - Acetic Acid - Water	85:5:15 70:15:30 60:30:10
5	Ethyl Acetate - Acetic Acid - Methanol - Water	60:15:15:10
6	n-Propanol - Water	70:30 85:15 90:10 95:5

Gas-Liquid Chromatography

1. Carbohydrates

a) Preparation of derivatives

i. Silylation (14): Approximately 50 mg of lyophilized wasted material and 1.0 ml of Tri-Sil (trimethylsilyl - pyrimidine mixture obtained from Pierce Chemical Co., P.O. Box 117, Rockford, Ill.) were introduced into a plastic stoppered vial and shaken vigorously for about 30 seconds. The mixture was allowed to stand for at least 5 minutes; one or two microliter quantities were then injected into the gas chromatograph. Column consisted of 3% SE-30 ultraphase on chromosorb W(HP) stainless steel 1/8 inch x 6 ft, (SE 30 General Electric Co.)

ii. Alditol Acetate Derivatives (modified method of Albersheim et al) (1): Ten mg of myo-inositol (internal standard) were added to 50 ml of $BaCl_2$ treated, centrifuged mill waste and the pH adjusted to about pH 6. Approximately 0.15 gm of sodium borohydride ($NaBH_4$) was added to reduce the sugars to their respective alcohols. After 30 min of reaction time at room temperature, the excess $NaBH_4$ was decomposed by the dropwise addition of glacial acetic acid until evolution of hydrogen gas ceased. The resulting boric acid was removed by evaporation in dryness or near dryness on a rotary evaporator "in vacuo". Ten ml of methyl alcohol was added, the mixture stirred and evaporated to dryness. This process was repeated two more times. The mixture was dried in an oven at 100 - 110°C for 10 minutes. Ten ml acetic anhydride and about 0.5 gm sodium acetate was added and the mixture refluxed at 140° for 20 min, then cooled slightly, removed from condensor and evaporated to dryness on a rotary evaporator. Five ml of dichloromethane were added, the mixture stirred, and centrifuged at 11,000 - 12,000 rpm for 20 min. Dichloromethane was decanted off into a test tube and evaporated to 1 ml. One or two microliter quantities were injected into the gas chromatograph for analysis.

b). Preparation of Column (8). One hundred mg ethylene glycol succinate (EGS) were dissolved in 25 ml of chloroform and 100 mg ethylene glycol adipate (EGA)

and 200 mg. XF 1150 were dissolved in 25 ml of acetone. The above mixtures were poured together quickly, mixed with 10 gm of gas chrom P, and stirred gently occasionally. After 1/2 hr, it was poured into a fritted funnel and excess liquid allowed to drain off until approximately 1 drop/30 sec. The mixture was poured into a large petri dish and dried. A 4' x 1/8" stainless steel column was filled by vibration (about 6cc.) and equilibrated in the GC for about 2-6 Hr at 180°C before used. It was advisable to flow a low level of helium gas through the column during equilibration (5 to 10 lb helium gas.) All analyses were made using a F & M High Efficiency Gas Chromatograph (Model 402) with a Honeywell Strip Chart Recorder (ElectroniK 16) and a Hewlett-Packard 3370A Integrator. The column was run isothermally at 180°C. Injection port and detector temperatures were 215°C and 210°C respectively. The approximate gas flow rates were as follows: helium 70 ml/min, hydrogen 40 ml/min, and air 230 ml/min. Two µl was the standard volume injected.

2. Volatile Fatty Acids, Alcohols

a. Sample Preparation (3): Waste liquor in the amount of 300 ml was adjusted to pH 10 with 3N NaOH in order to form the sodium salts of the volatile acids. The liquor was evaporated to dryness and the salts dissolved with 1.6 N H_2SO_4. Any remaining solids were filtered out with Whatman #1 filter paper. One or two microliter samples were injected into the gas chromatograph. Waste liquor samples could also be injected without treatment directly into the gas chomatograph.

b. Preparation of column. A 6' x 1/8" stainless steel column was filled by vibration with a packing mixture prepared as follows: Dissolve 1.0 gm of neopentyl-glycolsuccinate (NPGS) and 0.1 gm of concentrated phosphoric acid in about 50 ml of chloroform (enough to form a smooth slurry with Porapak Q). Once dissolved, mix with 10 gm 80-100 mesh Porapak Q and thoroughly stir the slurry in a small beaker. Evaporate to dryness and fill column by vibration. Equilibrate at 180°C for 6 to 12 hrs before using the column. It was advisable to flow a low level of an inert gas through column during equilibration (5 to 10 lbs. helium gas). Analyses were run on the same GC system described before in section 1b. Isothermal operation at 160°C was used.

Injection port and detector temperatures were 198°C and 195°C respectively. Gas flow rates were approximately as follows: helium 70 ml/min, hydrogen 20 ml/min and air 300 ml/min. The standard injection volume was 3 µl.

SECTION V

RESULTS AND DISCUSSION

Biological Analyses

It was apparent from the outset of this study that the experimental streams could not be used effectively to assess growth promoting properties of the wastes unless a constant flow could be established, and it was hoped that this could be achieved following the planned modification of the flow regulators. However, the modification never was completely successful. Initially, considerable effort was expended in modifying and improving a plate count procedure for the enumeration of Sphaerotilus in water. A tentative procedure had been developed but after preliminary testing it was felt that the procedure could be improved. A variety of media were tested for their ability to recover Sphaerotilus from water. These included CGT agar as described by Dondero (6) and various dilutions of Cytophaga agar.

In preliminary experiments a pure culture of Sphaerotilus was added to stream water and plated on the test media. The results of these studies are shown in Table 2. It can be seen that higher numbers of Sphaerotilus were obtained on dilute Cytophaga agar than on the richer media tested. On full strength Cytophaga agar and on CGT medium large numbers of eubacterial colonies developed and it is possible that these organisms inhibited the growth of Sphaerotilus. The results presented in Table 2 also show that a greater number of Sphaerotilus were recovered on 1/4 strength Cytophaga agar than on 1/10 strength Cytophaga agar. Furthermore, colonies on the 1/4 strength medium were somewhat larger and more easily counted.

Additional studies were carried out to test this medium for the recovery of Sphaerotilus from the natural environment. Samples of water were collected in sterile containers from the experimental streams receiving pulp mill wastes and were plated on both 1/4 CA and CGT agar. As shown in Table 3, 1/4 CA was more effective in recovering Sphaerotilus from natural samples than CGT medium. These findings confirm the earlier results.

The procedure described above appeared to be suitable for the recovery and enumeration of Sphaerotilus from natural bodies of water. Using this procedure it was possible to obtain reproducible results in triplicate plating tests. As shown in Table 4, each of the plates prepared from an individual water sample agrees closely with regard to the number of Sphaerotilus colonies present. It would be desirable to determine the reliability of this procedure by further testing with natural waters and to attempt to correlate plate count

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Table 2

Comparison of Media for the Isolation of Sphaerotilus

Medium	Number of Sphaerotilus/ml
CGT medium	150
Cytophaga agar	160
½ Cytophaga agar	600
1/10 Cytophaga agar	430

Table 3

Comparison of CGT and ½ Cytophaga Agar for Recovery of Sphaerotilus from Natural Waters

Medium	Number of Sphaerotilus/ml
CGY	0
½ Cytophaga agar	300

Table 4

Results of Triplicate Plating Tests Using ½ Cytophaga Agar

Sample	Plate 1	2	3
1	78[a]	71	74
2	32	32	28
3	31	35	28

[a]Number of *Sphaerotilus* colonies per plate

results with biomass data. It would seem that the number of free floating Sphaerotilus filaments and swarm cells in a stream should be proportional to the density of Sphaerotilus in that body of water. Data from the experimental stream studies which were planned should have provided information on this aspect. If the plate count procedure should prove to be a reliable technique for assessing the level of Sphaerotilus development in streams, it is possible that the procedure would have application in establishing water quality criteria and in monitoring the water quality of streams receiving pulp mill wastes or other wastes which promote Sphaerotilus growth.

A second approach which was planned to assess slime production in the experimental streams involved a slide technique. Clean glass slides were immersed into the stream for a period of time and the number of Sphaerotilus filaments which attached to the glass counted. Preliminary tests were carried out with this procedure in the experimental streams which had large populations of attached Sphaerotilus. It was found that more than a few hours of incubation in the stream produced slides so heavily laden with Sphaerotilus and silt that the filaments could not be counted. While the filaments attached to slides incubated in the stream a short time could be enumerated, it was felt that further attempts to standardize this procedure would be of little value until the streams became operative. Hence, no further experiments were carried out on this aspect of the study.

While the experimental stream facility could provide a worthwhile approach for assessing slime production, it requires accurate control of the waste added. Until it becomes possible to regulate the flow of wastes into the experimental streams reliably, this approach is of little value. For this reason no attempt was made to sample the streams on a regular basis.

The third approach for monitoring slime production involved the use of the continuous culture procedure. This method consisted of the addition of pulp mill waste, treated and untreated, at a constant rate to a continuous culture vessel inoculated with natural activated sludge organisms or Sphaerotilus.

After a known amount of waste passed through the culture vessel, the dry weight of the resulting growth was determined and an analysis of the residual sugars made. Preliminary experiments were carried out to standardize this procedure. Initially it was planned to use a dilute CGT broth as a basal medium for these studies. This medium proved to be unsatisfactory, however, since in some cases viability of the culture was lost before the addition of waste was initiated. In other cases the growth resulting was not filamentous and considerable wash-out occurred. A number of other basal media were tested and it

was found that 1/10 strength Cytophaga broth was the most satisfactory since filamentous attached growth resulted.

The procedure used to assess slime production by the continuous culture approach involved inoculating a culture vessel containing 500 ml of 1/10 strength Cytophaga broth with approximately 30 ml of a broth culture of Sphaerotilus. Growth was allowed to occur in the vessel for approximately 24 hours before the waste was introduced. This allowed the reserve material of the cells and the nutrients in the growth medium to be depleted before the addition of waste. Following this initial incubation period, 1/10 strength Cytophaga broth supplemented with a known volume of autoclaved waste was allowed to flow through the culture vessel at a rate of approximately 150 ml per hour. Incubation was at 25° C. The growth occurring in the vessel was collected, dried and weighed. While most of the growth remained in the culture flask, a small amount washed out in the effluent. For this reason the vessel effluent was collected in a container with potassium cyanide or acidified sodium hypochlorite and the dry weight of the cells determined. This was added to the cell yield obtained in the culture vessel.

In carrying out the analyses on the pulp mill wastes it was necessary to run a control culture vessel containing the basal medium alone. The amount of growth obtained in the control was subtracted from the cell yield obtained in the experimental flasks receiving the wastes. Also the amount of solids passing through the vessel was determined and subtracted from the cell yield. As a result, the cell yields reported for the different wastes represents the quantity of Sphaerotilus produced due to the nutrients in the wastes added.

Efforts to initiate the regular sampling of wastes was made the latter part of June, 1969. Unfortunately, it was not possible to obtain satisfactorily treated wastes at this time since difficulty was being experienced with the secondary treatment facility due to a malfunction in the aerators. Nevertheless, preliminary tests were performed on samples taken on two occasions in June. The cell yields obtained with these wastes are shown in Table 5. It is interesting to note that in the samples examined on 30 June, the cell yield from the secondary effluent was nearly the same as that obtained with the primary effluent. This would suggest either that the secondary influent waste sampled at this time was more dilute than that generally entering the secondary treatment facility or that the treatment was inefficient in removing nutrients which promote Sphaerotilus growth.

Regular sampling was begun in August after measures had been taken to correct the malfunctioning of the secondary treatment facility.

Table 5

Growth Promoting Properties of Pulp Mill Wastes as Determined by Continuous Flow Procedures

Sample Date	Type of Waste	Milligrams dry weight *Sphaerotilus* per liter waste
6/24/69	Secondary influent	96
6/30/69	Secondary influent	77
	Secondary effluent	75
8/6/69	Secondary influent	113
	Secondary effluent	53
8/12/69	Secondary influent	245
	Secondary effluent	120
8/22/69	Secondary influent	146
	Secondary effluent	69

Both secondary influent and secondary effluent samples were analysed at approximately weekly intervals. The results obtained with the samples analyzed in August also are shown in Table 5. It can be seen that the yield obtained with the secondary effluent was less than that obtained with the secondary influent collected at the same time. However, this does not necessarily represent the efficiency of the secondary treatment. A comparison between the growth promoting properties of the secondary influent and effluent wastes could be used to assess efficiency of the treatment only if the chemical composition of the secondary influent were relatively constant over an extended period of time. However, this does not appear to be the case when one notes the differences in the quantity of Sphaerotilus produced in the secondary influent obtained at approximately weekly intervals. Efficiency of the secondary treatment process could best be assessed with the continuous culture method by comparing the cell yields obtained with samples of effluent waste collected at regular intervals during successive treatment schedules. The number of samples which should be analyzed before the project was terminated was not sufficient to permit conclusions to be made regarding the effectiveness of a given treatment process. Nevertheless, this approach should provide a suitable means for assessing the efficiency of different methods of treatment.

Experiments were also carried out to test the possibility of using acidified wastes for analysis by the continuous culture procedure. The use of acidified wastes would permit samples to be collected and preserved for a period of time before analysis. The results obtained in four tests using acidified wastes was considerably different from those obtained with the corresponding fresh wastes. Hence acidification does not seem to be a satisfactory method for preserving wastes for subsequent analysis of their slime growth promoting properties. (The above results are those reported by Dr. R. E. Pacha, initial contract supervisor).

Chemical Analyses

As a means of identifying specific chemical factors which permit growth of Sphaerotilus, thin layer chromatography (TLC) and gas chromatography (GC) were used for determining the efficiency of carbohydrate removal in both the treatment ponds and continuous culture process.

Thin Layer Chromatography

These studies were qualitative rather than quantitative and were used in an effort to find a fast and efficient method of separating and identifying the sugars present in the mill waste.

The heavy trailing encountered during the first attempts to develop thin layer chromatograms could be minimized by precipitating the sulfates in the waste liquor with barium and by reconstituting the lyophilized solids with 95% ethyl alcohol. Although some sugars are only slightly soluble in alcohol, it was felt that the quantity of these sugars present in the waste liquor was sufficiently low to assure their complete solution.

Best results were achieved when chromatograms were developed twice in a solvent mixture of propanol and water (85:15, 70:30). As seen in Table 6 six bands could be distinguished. Visualization of the bands with ninhydrin showed a slight positive reaction from the origin up to and including Band I. Bands II through V showed R_f values established for the standard sugar mixture. Band III contained at least four bands so close together that no individual R_f values could be assigned. Although the color reactions of the standard sugars agreed with those of most bands in the region of 5.9 - 10.0 cm from the origin, no definite identification was possible because the R_f values were not consistent. Band VI, probably consisting of low molecular weight substances, was found to travel on the solvent front.

There appeared to be no detectable differences between chromatograms developed from effluent samples when based on the mode of operation of the aeration ponds (parallel vs. series operation). The cultures grown on untreated influent developed rapid growth with heavy slime formation. The mixed cultures which were grown on spent effluent developed slowly over a period of several weeks with slime formation along the inside wall of the culture vessel. The chromatograms, except for Bands IV and V appeared identical to those of the fresh influent.

Increasing the air supply to 2.0 CFH did not increase growth or alter the chromatographic picture. The fact that all particulate matter was removed from the effluent by centrifugation and filtration before being used as a substrate may be a partial explanation. Bacterial cells make up approximately 150 to 170 ppm of the total solids. These may recycle and serve as an excellent source of nutrients for Sphaerotilus and other microorganisms. When this biomass, which may constitute as much as 30% of the BOD, is removed as is done in the laboratory, the cultures lack sufficient nutrients for growth and/or utilization of residual carbohydrates.

The observations made thus far suggest that the quality of the paper mill effluent could be improved by more efficient aeration in the treatment ponds and by removal of the bacterial mass from the final effluent discharged.

Table 6

Band pattern of aeration pond influent on Silica Gel G developed twice in a solvent mixture of n-propanol and water (85:15,70:30).

Band No	Distance from Origin (cm)	Approx R_f
I	3.8 - 4.2	33
II	5.9 - 6.2	50
III	6.4 - 7.5	58
IV	8.0 - 8.2	67
V	9.3 - 10.0	78
VI	11.0 - 12.0	95

Gas Chromatography

The silylation procedure proved to be exceedingly fast and reproducible but for purposes of quantitation and even qualitative evaluation so confusing as to be impractical. Since silylation involves the conversion of hydroxy and polyhydroxy compounds into trimethylsilyl ethers, there may be as many as four peaks for each sugar due to anomerization in addition to the many peaks resulting from the silylation of phenols, organic acids, and certain amines. Consequently, the procedure was discontinued in favor of the relatively new alditol acetate approach which yields only one peak for each monosaccharide, thus making identification and quantitation feasible. The results of the initial attempts are shown in Figure 1 and 2.

Figure 2 represents the sugar analysis of an untreated influent sample entering aeration pond II and Figure 3 an effluent sample following treatment in pond II. It should be noted that ponds I and II were operating on a parallel schedule and the samples analyzed were grab samples rather than 24 hr composites. Based on retention times for standard sugars (Fig 5a) the carbohydrates in the influent (Fig. 2) were found to correspond with the retention times of the pentoses rhamnose, arabinose, and xylose, and the hexoses mannose, galactose, and glucose. Unidentified are some low molecular weight aldehydes or ketones which have a shorter retention time than shown for the standard (Fig 5 b,c,d).

A comparison of the chromatographed influent and effluent indicates that fermentable carbohydrates still remain following aeration during 7 days retention time. The six monosaccharides were usually present in varying amounts in both the treated and untreated effluent. However, not enough nutrients remain in the treated effluent to initiate or support growth of a suitable mixed flora. Nevertheless, over a period of seven days or more, Sphaerotilus will become established and continue to grow at a rate which becomes a problem at outfalls from paper mill plants.

Initial results with continuous mixed cultures inoculated into influent and effluent liquor are shown in Fig. 3 and 4 respectively. Growth is luxuriant on influent liquor and, in particular, the production of slime as Sphaerotilus. The microorganisms observed in such a culture are typical of those observed in a healthy bio-oxidation process. Controlled fermentation in the laboratory results in a more complete bio-oxidation of the influent carbohydrates than treatment in the aeration ponds.

Bacterial growth is limited when inoculated into filter sterile

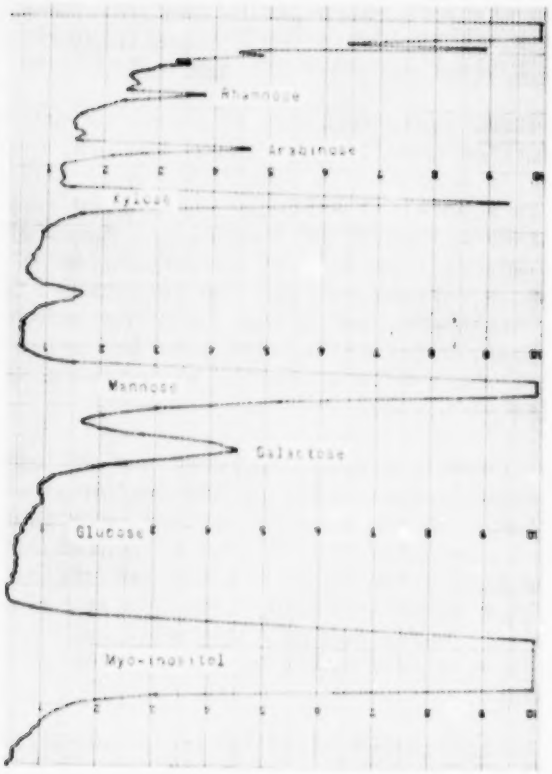

Figure 1. Gas chromatographic analysis of pentoses and hexoses present in the aeration pond influent.

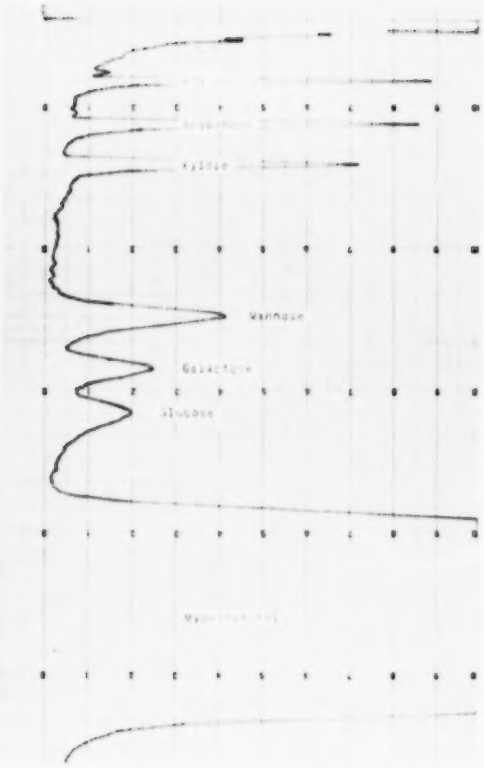

Figure 2. Gas chromatographic analysis of pentoses and hexoses present in the aeration pond effluent after treatment.

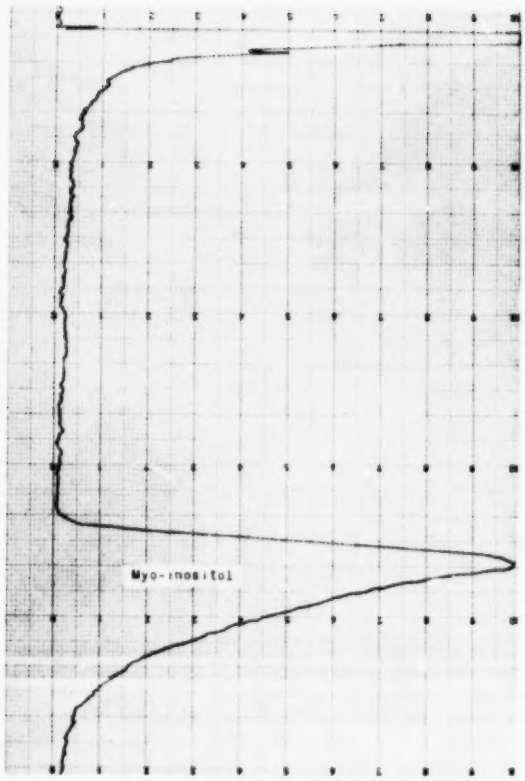

Figure 3. Gas chromatographic analysis of pentoses and hexoses in the spent liquor of continuous mixed cultures supplied with filter sterile aeration pond influent as substrate (500 ml per 24 hours and 2.0 CFH of air).

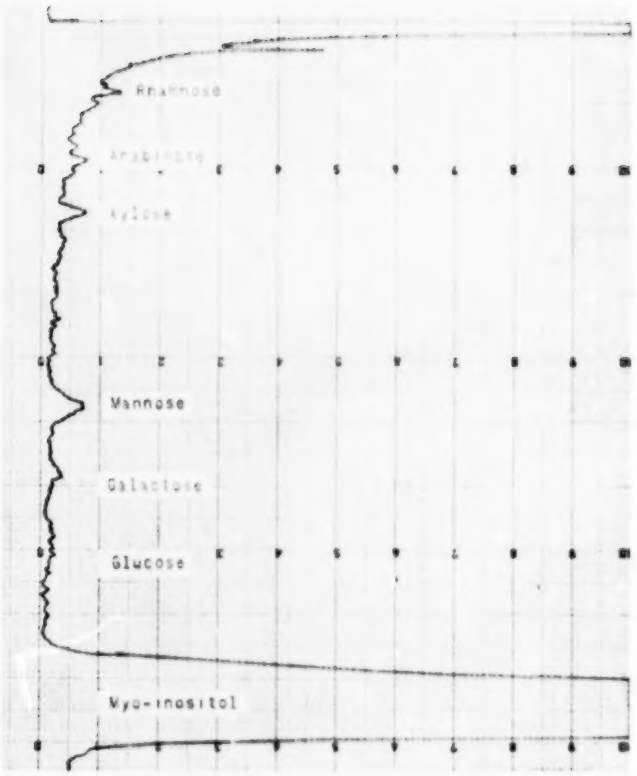

Figure 4. Gas chromatographic analysis of pentoses and hexoses in the spent liquor of continuous mixed cultures supplied with filter sterile aeration pond effluent as substrate (500 ml per 24 hours and 2.0 CFH of air).

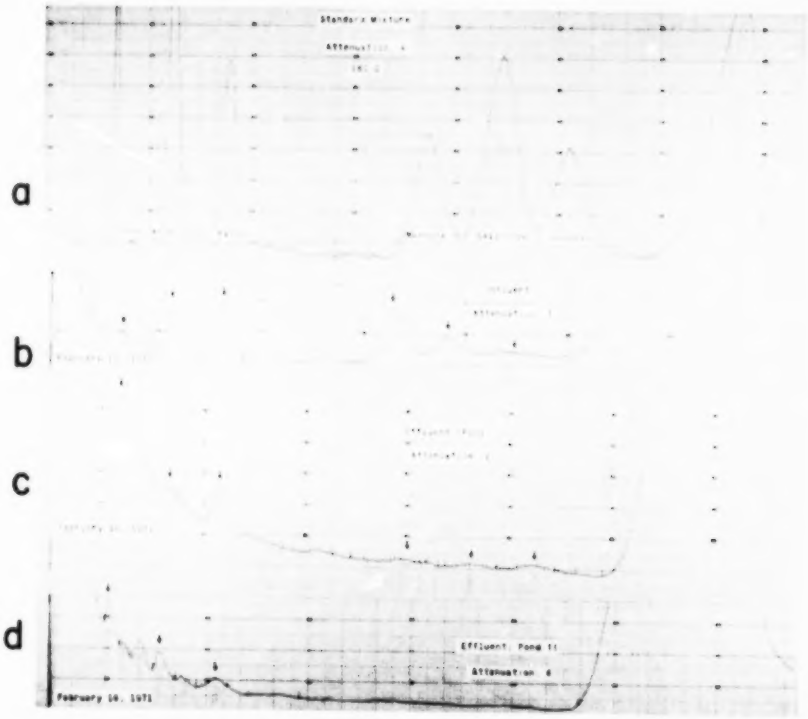

Figure 5. Gas chromatographic analysis of mill waste influent and effluents collected on February 16, 1971. (a) standard mixture of the six main wood sugars and the internal standard, myo-inositol, (b) influent; arrows left to right represent rhamnose, arabinose, xylose, mannose, galactose and glucose respectively, (c) effluent from aeration pond I with six wood sugars designated by arrows, (d) effluent from aeration pond II containing only pentoses; rhamnose, arabinose, and xylose as designated by arrows.

effluent. The negligible growth response is due to the fact that nutrients which support luxuriant growth have been depleted during the initial treatment process. However, Sphaerotilus will initiate growth over a period of 7 to 10 days. The fact that Sphaerotilus growth occurs at all supports the contention that bio-oxidation in the treatment ponds is not complete. Although the effluent BOD (Fig. 7) was within the established limits and did not fluctuate appreciably during the period of this research, Sphaerotilus grew to the exclusion of all other microflora in the effluent, as demonstrated both in the laboratory and in the outfall of the plant.

Figure 6 shows the continuous laboratory culture method using Spin Flasks adapted for continuous culture operation. The flask on the right in the photograph shows waste that was initially inoculated with a mixed activated sludge culture obtained from the Lebanon treatment pond. The substrate is a weekly sample of treated effluent. The turnover rate was approximately 24 hours. Although the flask was initially inoculated with a mixed culture as indicated above after a period of approximately 7 to 10 days Sphaerotilus becomes the predominating organism showing a gradual increase in filament mass over a period of a month. It would appear that there is sufficient energy for growth of Sphaerotilus, but not enough for other microbial species. Upon addition of a 0.1% glucose the microflora will revert to mixed culture typical of an activated sludge system.

The flask on the left in the photograph is fed a substrate consisting of the untreated weekly sample. The system was treated identically to the one on the right. The microflora remain unchanged over the several months of continuous culture. The substrates in both cases were filter sterilized to minimize unwanted biological changes during the test and to remove the biomass and other inert suspended solids which could serve as additional nutrients in the presence of the proper microbiological enzymes.

The GC analysis (Fig. 5,8,9, quantitated in Table 7,8,9) shows the amount of carbohydrates remaining in the secondary effluent which is sufficient to support the growth of Sphaerotilus. Weekly variations and differences between the two treatment ponds were observed in the residual monosaccharides (Fig. 13). Suggested reasons for these variations will be discussed later.

Laboratory treatment of the untreated influent showed that the sugars could be bio-oxidized below the level of detection by GC analysis within 24 hours (Fig. 3). Thus it would appear that the nitrogen, phosphorus, and other elemental and micronutrients were present in sufficient quantities to insure complete bio-oxidation. During the 8-month period that these tests were run, nitrogen in

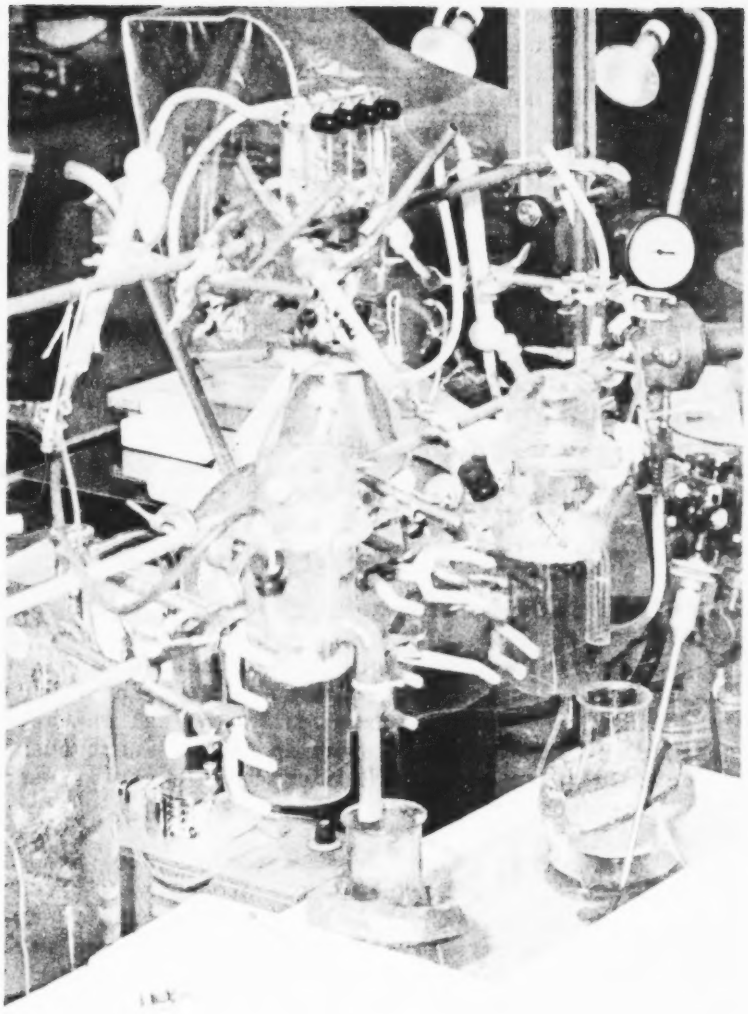

Figure 6. Continuous culture apparatus.

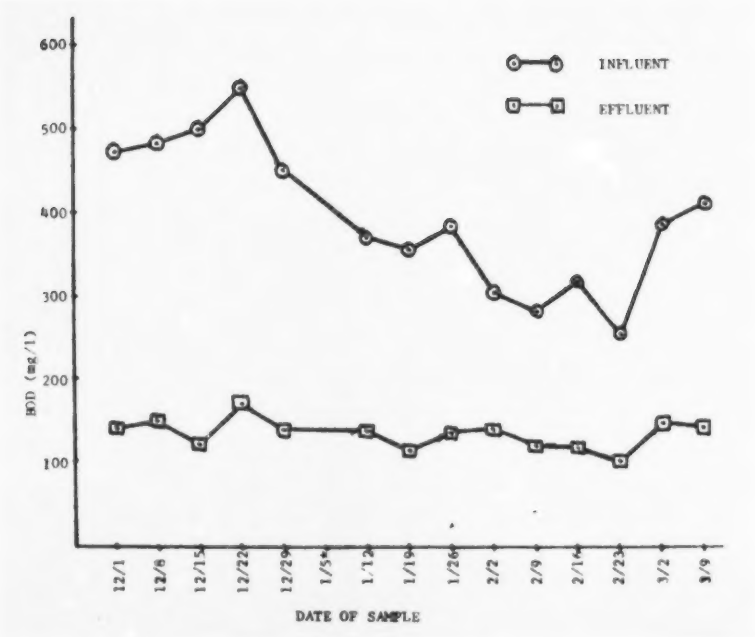

Figure 7. BOD+ of mill waste influent and effluent.

+BOD data obtained from daily log at Crown Zellerbach, Lebanon, Oregon.
*Data not available for this date.

Figure 8. Gas chromatographic analyses showing range of monosaccharide concentration in aeration pond I effluent. (a) highest sugar content, (b) average sugar content, and (c) least sugar content obtained during study. Arrows in b and c represent respective wood sugars labelled in a.

Figure 9. Gas chromatographic analyses showing range of monosaccharide concentration in aeration pond II effluent. (a) highest sugar content, (b) average sugar content, and (c) least sugar content obtained during study. Arrows in b and c represent respective wood sugars labelled in a.

Table 7

Approximate monosaccharide concentrations in aeration pond I effluent, 1970-1971.

Date	RHAMNOSE		ARABINOSE		XYLOSE		MANNOSE		GALACTOSE		GLUCOSE		TOTAL SUGARS**		***
	A*	B*	A	B	A	B	A	B	A	B	A	B	A	B	
11/24	.0002	1.7	.0010	8.3	.0008	6.7	.0002	1.7	.0003	2.5	.0368	306.4	.0704	586.2	55.8%
12/1	.0004	3.3	.0007	5.8	.0004	3.3	.0006	5.0	0	0	.0054	45.0	.1007	838.5	7.4%
12/8	.0006	5.0	.0006	5.0	.0009	7.5	.0008	6.7	0	0	.0064	54.4	.010	84.1	93.4%
12/15	.0003	2.5	.0005	4.1	.0014	11.6	.0027	22.5	.0011	9.2	.0008	6.7	.0099	82.4	68.6%
12/22	0	0	.0001	0.8	.0008	6.7	.0003	2.5	0	0	0	0	.0045	37.5	26.6%
1/5	.0005	4.2	.0002	1.7	.0019	15.8	.0003	2.5	0	0	.0002	1.7	.0527	438.8	5.9%
1/19	0	0	.0002	1.7	.0001	0.8	.0004	3.3	0	0	0	0	.0028	23.3	24.8%
1/26	0	0	0	0	.0006	5.0	.0062	51.6	0	0	0	0	.0080	66.6	84.9%
2/2	0	0	.0002	1.7	.0001	0.8	.0014	11.6	0	0	0	0	.0028	23.3	60.5%
2/9	.0002	1.7	0	0	0	0	0	0	0	0	0	0	.0021	17.5	9.7%
2/16	.0002	1.7	.0001	0.8	.0011	9.2	.0001	0.8	.0001	0.8	0	0	.0017	14.2	93.6%
2/23	0	0	0	0	.0028	23.2	0	0	0	0	0	0	.0032	26.6	87.2%
3/2	.0014	11.6	0	0	.0007	5.8	0	0	0	0	0	0	.0029	24.1	72.1%
3/9	0	0	0	0	.0004	3.3	0	0	0	0	0	0	.0048	40.0	8.2%

*
A* = grams per liter, approximately
B = pounds per million gallons, approximately
**Total sugars indicates the concentration of the six identified sugars plus other unidentified monosaccharides observed on the gas chromatograph
***Percent monosaccharides identified

Table 8

Approximate monosaccharide concentrations in aeration pond II effluent, 1970-1971.

Date	RHAMNOSE		ARABINOSE		XYLOSE		MANNOSE		GALACTOSE		GLUCOSE		TOTAL SUGARS**		***
	A*	B*	A	B	A	B	A	B	A	B	A	B	A	B	
11/24	0	0	.0001	0.8	0	0	0	0	0	0	.0305	254.0	.0601	500.4	50.9%
12/1	.0009	7.5	.001	0.8	.0002	1.7	.0004	3.3	0	0	.0070	58.3	.0500	416.4	17.1%
12/8	.0004	3.3	0	0	0	0	.0005	4.2	.0003	2.5	.0022	18.3	.0038	31.6	89.5%
12/15															
12/22	0	0	.0002	1.7	.0001	0.8	.0012	10.0	.0003	2.5	0	0	.0019	15.8	94.9%
1/5	.0004	3.3	0	0	0	0	.0020	16.6	0	0	0	0	.0115	95.8	20.7%
1/19	0	0	.0005	4.2	.0005	4.2	0	0	0	0	0	0	.0013	10.8	77.7%
1/26	0	0	0	0	.0006	5.0	.0003	2.5	0	0	0	0	.0007	5.8	129.3%
2/2	0	0	0	0	.0001	0.8	.0016	13.3	0	0	0	0	.0022	18.3	77.0%
2/9	.0005	4.2	0	0	.0002	1.7	0	0	0	0	0	0	.0063	52.5	11.2%
2/16	.0003	2.5	.0003	2.5	.0003	2.5	0	0	0	0	0	0	.0016	13.3	56.3%
2/23	.0001	0.8	0	0	.005	4.2	0	0	0	0	0	0	.0009	7.5	66.6%
3/2															
3/9	.0003	2.5	.0001	0.8	.0033	27.5	0	0	.0001	0.8	.0001	0.8	.0016	13.3	243.6%

*A = grams per liter, approximately
B = pounds per million gallons, approximately
**Total sugars indicates the concentration of the six identified sugars plus other unidentified monosaccharides observed on the gas chromatograph
***Percent monosaccharides identified

Table 9

Approximate monosaccharide concentrations in aeration pond influent, 1970-1971.

Date	RHAMNOSE		ARABINOSE		XYLOSE		MANNOSE		GALACTOSE		GLUCOSE		TOTAL SUGARS**		***
	A*	B*	A	B	A	B	A	B	A	B	A	B	A	B	
11/24	--		--		--		--		--		--		--		--
12/1	.0004	3.3	.0055	45.8	.0103	85.8	.0174	144.9	.0058	48.3	.0123	102.4	.0673	560.4	76.8%
12/8	.0018	15.0	.0037	30.8	.0063	52.5	.0155	129.1	.0072	60.0	.0024	20.0	.1321	1100.0	27.9%
12/15	.0003	2.5	.0013	10.8	.0034	28.3	.0074	61.6	.0028	23.3	.0009	7.5	.0591	492.1	27.2%
12/22	.0012	10.0	.0085	70.8	.0126	104.9	.0347	288.9	.0120	99.9	.0051	42.5	.0799	665.3	92.7%
1/5	.0003	2.5	.0023	19.2	.0051	42.5	.0032	26.6	.0021	17.5	0	0	.1550	1290.7	8.3%
1/19	0	0	.0020	16.6	.0028	23.3	.0015	12.5	.0019	15.8	0	0	.0132	109.9	62.0%
1/26	0	0	.0048	40.0	.0062	51.6	.0073	60.8	.0030	25.0	0	0	.0231	192.4	92.2%
2/2	0	0	.0026	21.6	.0041	34.1	.0096	79.9	.0018	15.0	0	0	.0190	158.2	95.2%
2/9	0	0	.0025	20.8	.0066	55.0	.0033	27.5	.0016	13.3	.0009	7.5	.0196	163.2	76.0%
2/16	0	0	.0039	32.5	.0060	50.0	.0123	102.4	.0077	64.1	.0005	4.2	.0373	310.6	81.5%
2/23	0	0	.0021	17.5	.0022	18.3	.0025	20.8	.0017	14.2	.0003	2.5	.0099	82.4	88.9%
3/2	.0003	2.5	.0059	49.1	.0383	318.9	.0005	4.2	.0017	14.2	.0098	81.6	.0574	478.0	98.4%
3/9	.0009	7.5	.0022	18.3	.0046	38.3	.0051	42.4	67.0024	20.0	0	0	.0165	137.4	92.1%

*A = grams per liter, approximately
B = pounds per million gallons, approximately
**Total sugars indicates the concentration of the six identified sugars plus other unidentified monosaccharides observed on the gas chromatograph
***Percent monosaccharides identified

the form of ammonia and phosphorus in the form of phosphoric acid was added in excess daily. The pH of the influent was approximately 7.3. However, this could vary over a range of 4.3 to 9.5 (Table 10), while the treated effluent was fairly constant at about 6.8 (Table 10). The daily inflow varied from 3.77 MGD to 6.17 MGD (Fig. 10). The temperature varied from 13°C to 22°C over the same period (Table 11). The retention time depending upon the inflow rate was approximately seven days. Only trace amounts of acetic acid and methyl and ethyl alcohols were detected, if at all, by GC analysis of the secondary influent and effluents.

The factors which appeared to have the greatest effect on the efficiency of the treatment system are the influent flow rate (Fig. 10) and the rate of aeration. If the inflow rate (Fig. 10) is compared with total carbon (Fig. 11), total suspended solids (Fig. 12) and total monosaccharides (Fig. 13), it can be observed that inflow increases generally coincide with increases in suspended solids, total carbon, and monosaccharide concentrations in both the influent and effluent wastes. The occurence of these marked changes was not reflected in changes in the effluent BOD (Fig. 7). This would indicate that reliance on BOD as the only indicator of the efficiency of treatment could lead to an erroneous conclusion.

The present design parameters as well as the capacity are approximately identical. The only difference between the two ponds is the aeration systems. Treatment pond II was aerated by two 75 HP aerators while pond I was aerated by six 25 HP units. The horsepower input on both was identical. Thus the two 75 HP units appear to do a more effective aeration job resulting in a better biodegradation.

Several factors may account for the incomplete oxidation observed. The placement of the aerators may be such that not all the influent waste is subjected to sufficient aeration due to short circuiting, and appears in the effluent discharge. The aerators may not have the capacity to properly aerate the intermittent surges which contain an abnormally high content of biodegradable substances. A properly designed pond could insure that the retention time of all volumes and levels of waste solids was such that the carbohydrates are completely bio-oxidized. This would probably involve a redesign of the treatment ponds, increased aeration efficiency or capacity, or a different placement of the aerators.

Table 10

pH values of aeration pond influent and effluent over a 9-month period.

DATE AND SAMPLE	AVERAGE pH	RANGE	
		LOW	HIGH
July, 1970			
Influent	7.7	6.6	8.8
Effluent	7.0	6.7	7.4
August			
Influent	7.6	7.2	8.0
Effluent	6.6	5.8	7.3
September			
Influent	5.2	4.3	9.5
Effluent	6.9	6.5	7.1
October			
Influent	8.4	7.6	9.6
Effluent	6.6	6.1	6.9
November			
Influent	7.2	6.5	7.6
Effluent	6.8	6.1	7.2
December			
Influent	7.9	7.0	9.5
Effluent	6.4	5.9	7.2
January, 1971			
Influent	7.2	7.1	7.4
Effluent	5.9	5.9	5.9
February			
Influent	7.2	6.8	8.3
Effluent	6.3	5.8	6.8
March			
Influent	7.6	7.1	8.2
Effluent	6.4	5.9	6.9

Table 11

Temperature values of aeration pond influent and effluent over a 9-month period.

DATE AND SAMPLE	AVERAGE TEMPERATURE °C	RANGE	
		LOW	HIGH
July, 1970			
Influent	35	35	35
Effluent	27	27	27
August			
Influent	35	33	36
Effluent	27	26	27
September			
Influent	30	21	35
Effluent	28	19	25
October			
Influent	29	29	29
Effluent	21	18	23
November			
Influent	28	23	32
Effluent	20	18	21
December			
Influent	19	16	23
Effluent	16	14	17
January, 1971			
Influent	25	24	26
Effluent	16	16	16
February			
Influent	29	25	32
Effluent	21	19	22
March			
Influent	23	19	27
Effluent	16	16	17

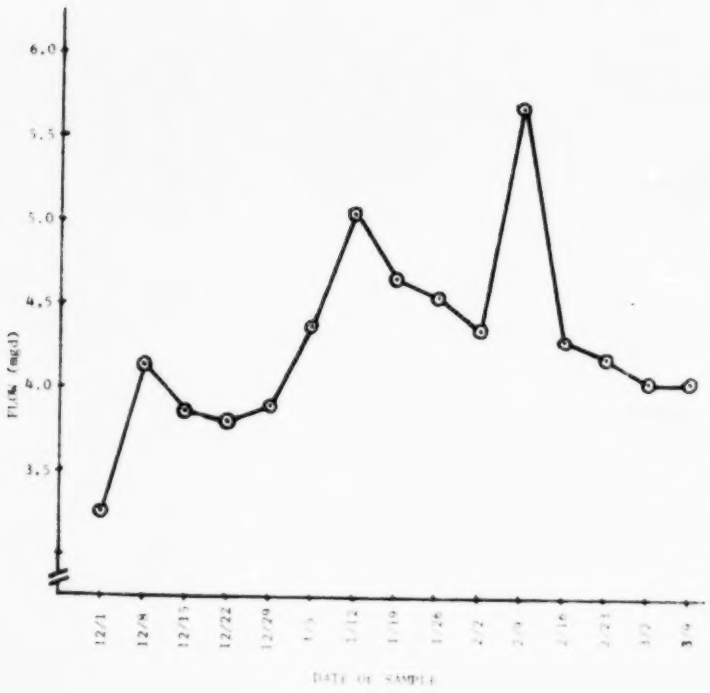

Figure 10. Daily flow rate* of mill wastes

*Flow rate data obtained from daily log at Crown Zeilerbach, Lebanon, Oregon.

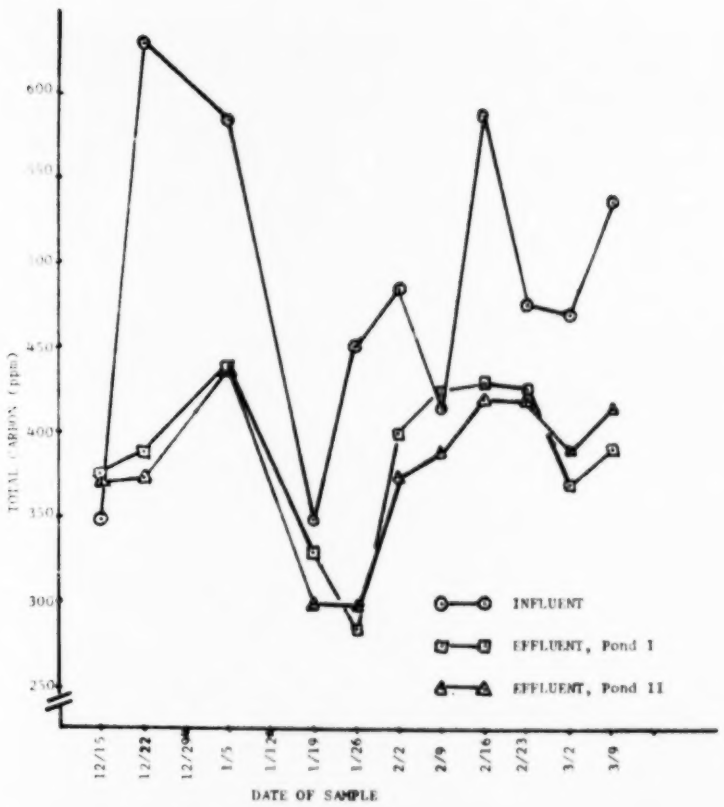

Figure 11. Total carbon concentration in filter sterilized mill waste influent and effluents.

*Data not available for these dates.

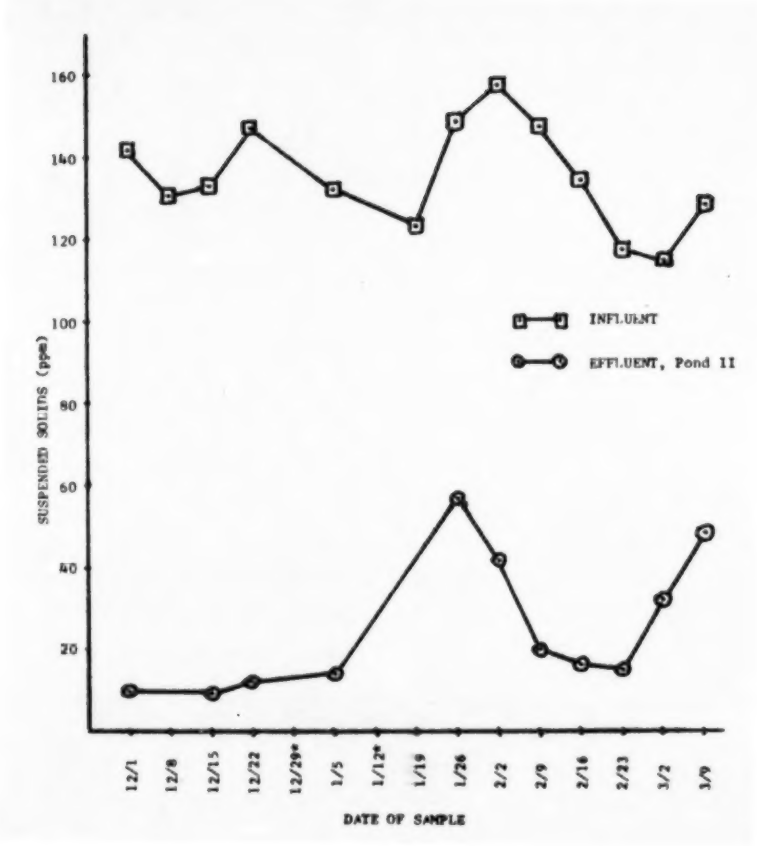

Figure 12. Suspended solids concentration in mill waste influent and effluent grab samples.

*Data not available for these dates.

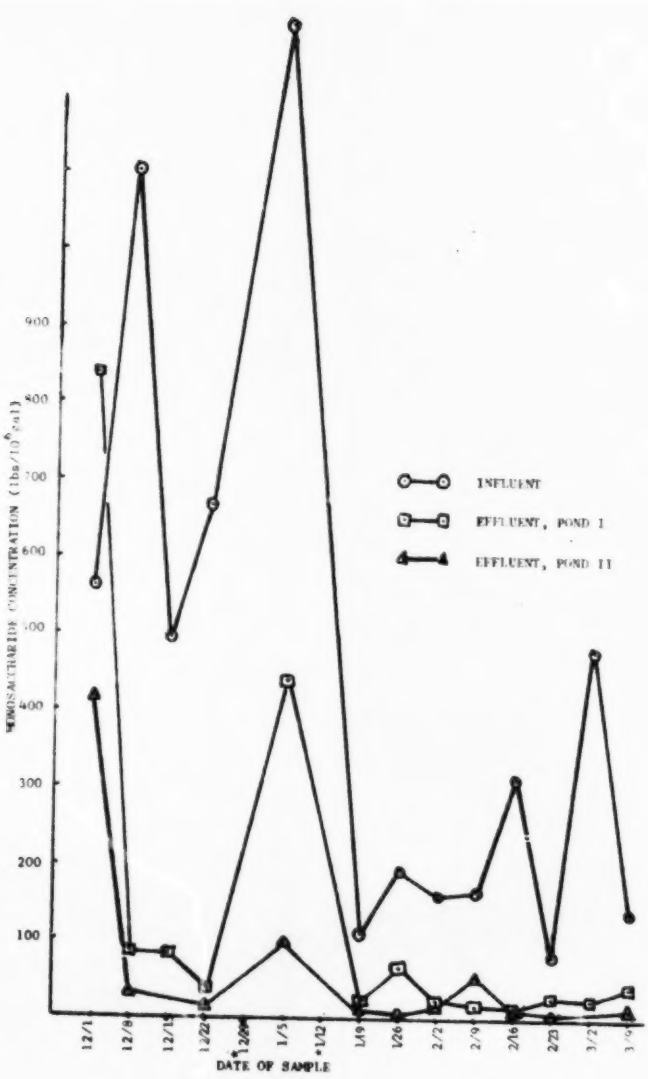

Figure 13. Estimated total monosaccharide concentration in mill waste influent and effluent composite samples.

*Data not available for these dates.

SECTION VI

ACKNOWLEDGMENTS

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SECTION VII

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SECTION VIII

GLOSSARY

Sphaerotilus - A sheathed bacteria commonly found in large slime masses in flowing waters where organic pollution exists.

BOD - Biochemical oxygen demand is the amount of oxygen utilized during microbial degradation or oxidation of organic material. With large amounts of organic material, waters may be totally depleted of free oxygen due to this phenomenon.

COD - Chemical oxygen demand measures the oxygen equivalent of organic matter which is susceptible to oxidation by a strong chemical oxidant.

Biodegradable - Substances able to be broken down by living organisms.

Influent - Mill wastes which flow into treatment ponds.

Effluent - Wastes liquors which flow out of treatment ponds.

Continuous culture - A system where substrate is continuously fed into a culture vessel of a specific volume. Spent substrate and cells flow out at the same rate, thus keeping the volume constant. The vessel culture may be aerated, cooled or heated, pH adjusted, dissolved oxygen measured, etc.

Grab sample - A sample collected at one time and from one location, thus not truely representing over-all conditions.

Lyophilized - This term is synonymous with freeze-drying; a process which depends on extreme cold and vacuum. Water is removed by passing from the solid to the vapor state without melting.

Biomass - The living organisms which are found in a particular system.

R_f value - This value represents the distance traveled by each compound from the origin relative to the solvent front, or

$$R_f = \frac{\text{distance from base line traveled by compound.}}{\text{distance from base line traveled by solvent}}$$

The R_f value is characteristic of a particular compound measured under specific conditions such as; solvent system, temperature, manner of development, type of paper or layer, and the grain direction of the paper or layer.

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TLC bands - During TLC, a compound will move a specific distance from the origin forming a spot or band. This band is characteristic of only that compound under the specific conditions of the run. Thus, two distinct and sharp bands in TLC would represent two different compounds.

Pentoses - Monosaccharides containing five carbon atoms.

Hexoses - Monosaccharides containing six carbon atoms.

Short circuiting - Influent bypassing aerators without adequate aeration resulting in a partially treated discharge.

Retention time - The time from sample injection into a system, such as in GC or aeration ponds, until the same sample leaves the system.